Hydrogen sulfide

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CHAPTER

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OVEREXPRESSION OF CYSTATHIONINE γ-LYASE SUPPRESSES SPINOCEREBELLAR ATAXIA TYPE 3-ASSOCIATED NEURODEGENERATION

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ABSTRACT

Spinocerebellar ataxia type 3 (SCA3) is a polyglutamine (polyQ) disorder caused by a CAG repeat expansion in the ataxin3-gene resulting in toxic protein aggregation. There is no cure that halts or reverses the symptoms of this devastating disease. The transsulfuration pathway has been implicated in crucial physiological processes, including those in the central nervous system. Activation of this pathway leads to the biosynthesis of the physiologically active gas hydrogen sulfide (H_2S), which is endogenously produced by cystathionine γ-lyase (CSE). H_2S has anti-oxidative and anti-inflammatory properties, making it an attractive candidate to intervene in the pathogenesis of SCA3. We investigated a possible modifying role of CSE in SCA3. We demonstrated that CSE is expressed in vascular endothelium, neurons and astrocytes of human pontine tissue. The CSE localization pattern is not affected in SCA3 patients, however CSE expression levels are decreased, suggesting a possible mediating role of CSE in SCA3 induced neurodegeneration. We demonstrated that CSE overexpression in *Drosophila* suppresses SCA3-associated degeneration. This decrease in degeneration does not coincide with decreased levels of insoluble protein aggregates but is associated with reduced oxidative stress and a dampened immune response. Treatment of SCA3-bearing flies with the endogenous H_2S donor sodium thiosulfate, a clinically used compound, resulted in similar protective effects. Our data indicate that the beneficial effects of CSE overexpression are due to protection against effects downstream of toxic protein aggregates and because of an increased production of H_2S. Together, the data implicate a modifying role of the CSE/H_2S-axis in SCA3 induced tissue degeneration.

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INTRODUCTION

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease, is a progressive neurodegenerative disease and the most common dominantly inherited ataxia worldwide. SCA3 is a polyglutamine (polyQ) disease caused by a CAG-trinucleotide repeat expansion encoding glutamine within the sequence of the ATXN3 gene. Expansion of the glutamine repeats results in aggregation of the ataxin3 protein leading to neuronal dysfunction and cell death.[1-3] The pathophysiological sequel of neurodegeneration in SCA3 is not fully understood, although proteotoxic stress, transcriptional dysregulation, mitochondrial dysfunction, oxidative stress and inflammation have been previously implicated.[4-6] To date in the clinic, there are no disease-modifying treatments for polyQ diseases like SCA3.

The transsulfuration pathway has been increasingly linked to aging and age-related pathologies.[7-10] Alongside cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST), cystathionine γ-lyase (CSE) is one of the important enzymes in the sulfur production pathway (Fig. 1). A key biological product of CSE is hydrogen sulfide (H_2S), an antioxidant and neuroprotectant small molecule gaseous transmitter. It is synthesized from L-cysteine and D-cysteine by CSE, CBS and 3-MST.[11-13] H_2S is then serially oxidized to persulfide, thiosulfate (TS, S_2O_3), sulfite and sulfate (Fig. 1). TS is an intermediate of sulfur metabolism from cysteine and a metabolite of H_2S that can also lead to the production of H_2S through the action of thiosulfate reductase[14] (Fig. 1). H_2S can act as an endogenous modulator of oxidative stress either by direct scavenging of reactive oxygen species (ROS), or through increasing the intracellular glutathione (GSH) pool.[15,16] H_2S also confers cytoprotection via suppression of inflammation and apoptosis,[17] and by protecting mitochondrial function and integrity.[17,18] Decreased levels of H_2S in brain tissue are associated with neurodegenerative age-related diseases like Parkinson's[19] and administration of H_2S has been shown protective in experimental models for this disease.[20,21] Decreased levels of CSE have recently been observed in Huntington's disease tissues and in a mouse Huntington model.[22] A possible link between SCA3 and CSE and potential neuroprotective effects of overexpressing the CSE enzyme directly remain to be determined.

The beneficial effects of H_2S on inflammation and oxidative stress together with its connection to age-related neurodegenerative diseases, led us to investigate a possible role of CSE in SCA3. First we showed that CSE is present in brain areas affected in SCA3 patients and in the latter CSE levels are decreased. Next, we investigated the protective effects of induction of the transsulfuration pathway in a SCA3 disease model. Because the H_2S-producing pathway is highly conserved in Drosophila melanogaster and expression of a truncated version of the pathogenic human ATXN3 gene containing a multiple CAG repeat (further referred to as SCA3 flies) in this organism recapitulates key features of SCA3 disease,[23,24] we used this fly model to investigate the protective effects of CSE overexpression. We found that transgene-mediated increased gene expression of CSE and addition of an endogenous donor
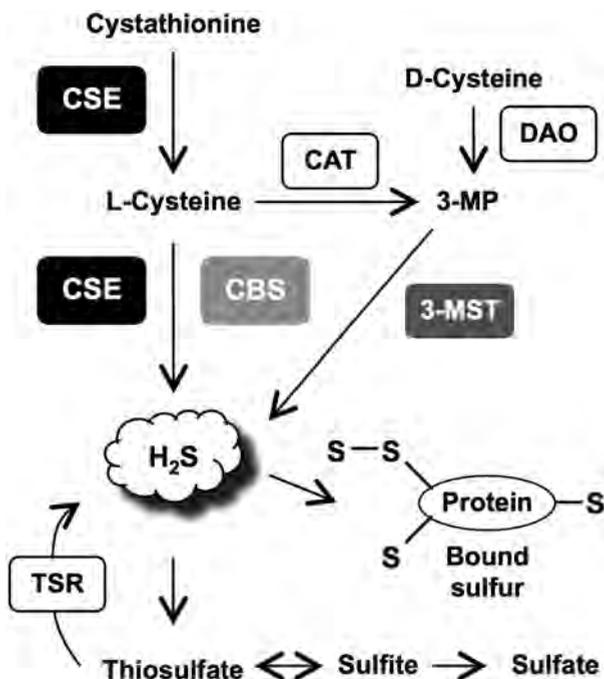
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Figure 1 – Schematic representation of endogenous H$_2$S production by CSE. Part of the transsulfuration pathway is presented. CSE converts cystathionine to L-cysteine and L- cysteine to H$_2$S. L-cysteine and D-cysteine are converted to 3-MP (3-mercaptopyruvate), which is then transformed to H$_2$S by 3-MST. Subsequently, H$_2$S is metabolized into thiosulfate. Thiosulfate is converted to H$_2$S by TSR (thiosulfate reductase) or can be oxidized to sulfite and then to sulfate. H$_2$S reacts with proteins and sulfhydrates them to form a bound sulfur pool. CAT, cysteine aminotransferase; DAO, D-aminoacidoxidase.

of H$_2$S partially rescued the phenotype of SCA3 in Drosophila downstream of protein aggregate formation. Rescue is associated with decreased levels of oxidative proteins and a dampening of the immune response. Last, we present and discuss a possible clinical relevance of CSE in SCA3 disease.

RESULTS

Levels of CSE are reduced in brain tissue of SCA3 patients

In order to investigate a possible role of CSE in SCA3 pathogenesis we investigated expression levels and localization of CSE in healthy tissue and in SCA3 disease tissue. To determine the presence and localization of CSE, we performed immunohistochemistry for CSE on post-mortem pontine tissue from patients without a neurodegenerative or neuropsychiatric disease (Supplementary Table 1, n=7). CSE protein expression was observed in vascular endothelium, neurons and astrocytes (Fig. 2A-2F). This localization pattern was not affected in pontine tissue of SCA3 patients (Supplementary Table 1, n=6) To enable quantification of the amount of CSE, we performed qPCR and Western

blot analyses for CSE on pontine samples of SCA3 patients and control samples. qPCR data revealed decreased mRNA levels of CSE in the pontine tissue from SCA3 (n=6) patients compared to control tissue (n=7) (Fig. 2G). Likewise, Western blot analysis showed reduced CSE protein levels in pontine tissue from SCA3 patients (n=4) (Fig. 2H). These results show that CSE is present in human brain tissue from SCA3 patients and that SCA3 is associated with reduced levels of CSE. Recently decreased levels of CSE were also observed in stratal brain samples from patients with Huntington's disease.[22]

Generation and characterization of various CSE transgenic lines

To further investigate a possible modulating role of CSE in SCA3 pathogenesis we used the model organism *Drosophila melanogaster*. *Drosophila* has been used in a wide variety to understand basic principles of human diseases.[25,26] Eip55E is a *Drosophila* ortholog of the human CSE gene.[27] Six different fly lines overexpressing Eip55E (further referred to as CSE) under a GAL4-inducible promoter were created. CSE expression
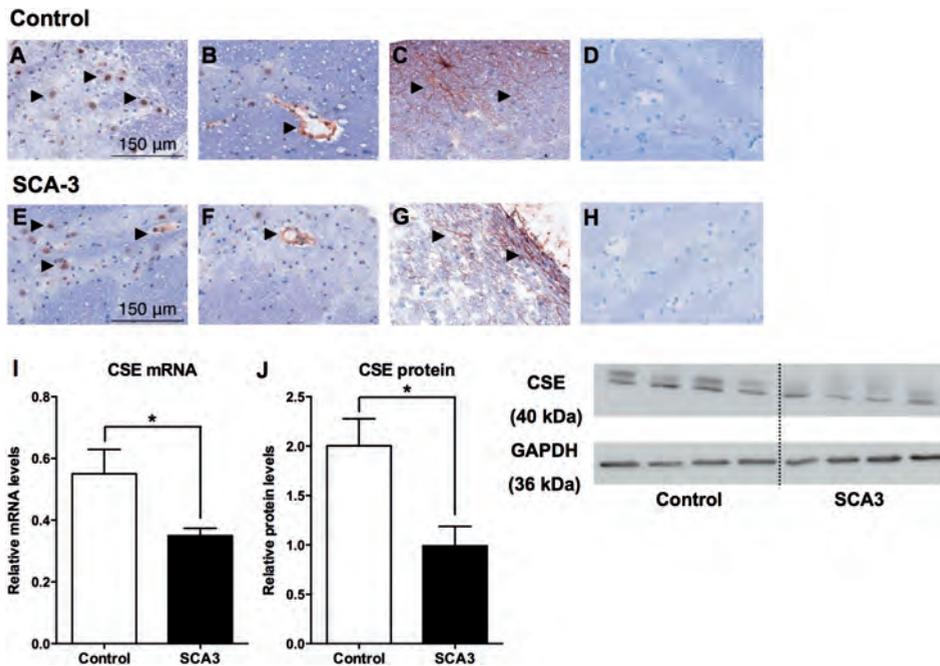

Figure 2 – CSE levels are decreased in brains of SCA3 patients. Immunohistochemistry revealed that in human pontine tissue, CSE is localized in (A and E) neurons of the pontine nuclei, (B and F) the vasculature and (C and G) astrocytes. Black arrows indicate the mentioned structures. No differences in staining pattern were observed between control and SCA3 brain tissue. Negative controls showed no staining, representative images are shown (D, H). Scale bar indicates 150 μm in all images. (I) CSE mRNA (control n=7 (lane 1-4); SCA3 n=6) and (J) protein levels were decreased in pontine tissue of SCA-3 patients compared to control brain tissue, 4 representative SCA3 samples are visualized (lane 5-8) and quantified. *p<0.05, error bars indicate SEM.

levels of each line were determined in the presence of a daughterless driver resulting in ubiquitous expression of the protein. All six lines showed increased expression of CSE compared to the in-house control w1118 strain (Supplementary Fig. 1A). As the genetic background of *Drosophila* plays an important role in the severity of specific phenotypes,[28] all transgenic overexpressing lines were backcrossed for at least 6 generations to create isogenic controls. As an isogenic control, one strain (strain 1, Supplementary Fig. 1) was backcrossed with the w1118 control strain that was used to generate the transgenic lines. The CSE strain 1 overexpressing line is further referred to as CSE-1A and its isogenic non-CSE-expressing control line is further referred to as w1118-A. Other lines (CSE-2 to CSE-6) were backcrossed with the in-house w1118 strain to generate an isogenic control line. From these, two lines were selected: one line overexpressing CSE to a lower level (further referred to as CSE-2B) and one line overexpressing CSE to a higher level (further referred to as CSE-3B) (Supplementary Fig. 1). The isogenic control of these lines is further referred to as w1118-B. This approach allowed us to compare the effect of CSE overexpression in 2 genetic backgrounds, to investigate the effect of variations in overexpression levels, and to compare this to isogenic controls. By using qPCR, we demonstrated that CSE-1A showed a 2.1-fold induction of CSE compared to its isogenic control, and that CSE-2B and CSE-3B showed a 2.2-fold and a 5.3-fold increased expression of CSE compared to their isogenic control, respectively (Fig. 3A and 3B).

Overexpression of CSE partially rescues the phenotype of SCA3 in Drosophila

Previously, it has been shown that flies bearing *UAS-SCA3trQ78* – a truncated version of the human ATXN3 gene containing 78 CAG repeats – under the control of the glass multiple reporter (GMR) driver (also referred to as SCA3 flies) develop progressive
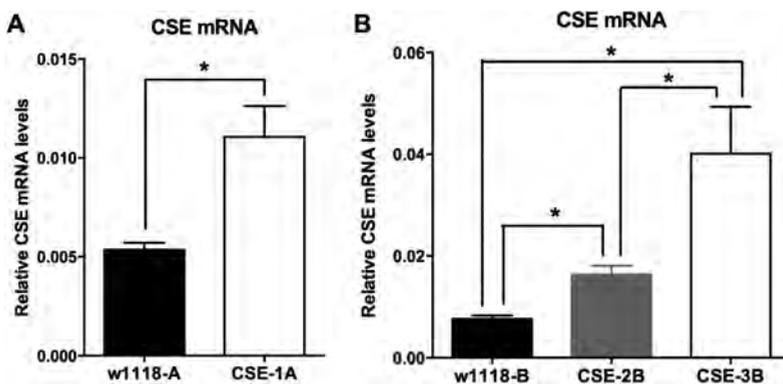

Figure 3 – **Increased CSE mRNA levels in CSE overexpression flies in two different genetic backgrounds.** (A and B) CSE overexpression was confirmed with qPCR in all three transgenic lines. In both genetic backgrounds CSE mRNA levels were increased in the CSE overexpressing lines (CSE-1A, CSE-2B, CSE-3B) compared to their isogenic controls. *$p < 0.05$, error bars indicate SEM.

cellular eye degeneration. These flies develop a 'rough eye' phenotype and a certain percentage of the rough-eyed flies possess a variable amount of patches with increased degeneration.[23,24] Rough eyes containing these degenerative patches (further referred to as degenerated rough eyes) are considered to be more affected compared to the rough eyes only, and are a useful tool to study enhancers or suppressors of the SCA3-induced toxicity.[29] To visualize these phenotypic differences at a higher magnification, we performed correlative light microscopy and scanning electron microscopy on the eyes of wild type flies (Fig. 4A) and flies overexpressing human ATXN3 (Fig. 4B-C). It appeared that at the rough eyes ommatidia and bristle structures can be observed (Fig. 4B-B") and, in contrast, the degenerative patches consist of an undefined structure and bristles are absent (Fig. 4C-C"). These results confirm that degenerative patches can indeed be classified as more severely affected. In the SCA3 background, CSE-overexpressing flies showed a significant decrease in the percentage of degenerative rough eyes. As a control, SCA3 flies were crossed with the isogenic wild type control lines. For quantification, we scored the number of degenerated eyes at day 1 after eclosion. Suppression of the SCA3 phenotype was observed in all CSE-overexpressing lines compared to their isogenic control lines. Similar results were obtained in both genetic backgrounds (Fig. 4D and 4E). The CSE-3B line with the highest level of CSE overexpression reduced the number of degenerative eyes to a greater extent than the CSE-2B line (Fig. 4E). To further strengthen the rescue potential of CSE, we pharmacologically inhibited CSE with propargylglycine (PPG) as previously described.[7] Supplementation of PPG to the fly food reversed the protective effect of CSE overexpression as evidenced by an increased percentage of degenerated rough eyes (Fig. 4D and 4E). These results further indicate that the rescuing potential is mediated by CSE. To exclude that the suppression of SCA3 in the CSE transgenic lines is due to a GAL4 titration effect using multiple UAS constructs in one genotype,[30] we crossed the GMR-GAL4-UAS-SCA3 flies with a fly line containing a UAS green fluorescent protein (UAS-GFP) construct and two fly lines containing a different yellow fluorescent protein (UAS-YFP) construct. If the suppression of eye degeneration as observed in the CSE-overexpressing SCA3 flies was due to the titration of GAL4, the percentage of degenerated eyes would also be reduced in the progeny of these crosses. However, in the crosses with the GFP line and both YFP lines a similar degree of eye degeneration was observed as compared to the isogenic wild type controls line of these lines (Supplementary Fig. 2). This indicates that the suppression of the SCA3-induced degeneration is CSE-specific and is not just caused by expression of a UAS-bearing construct.

Treatment with sodium thiosulfate reduces eye degeneration in SCA3 flies

To further investigate the protective mechanism of CSE overexpression, we treated GMR-GAL4-UAS-SCA3 flies with increasing concentrations of sodium thiosulfate (STS). Thiosulfate (TS) is an endogenous intermediate of the sulfur metabolism, which can
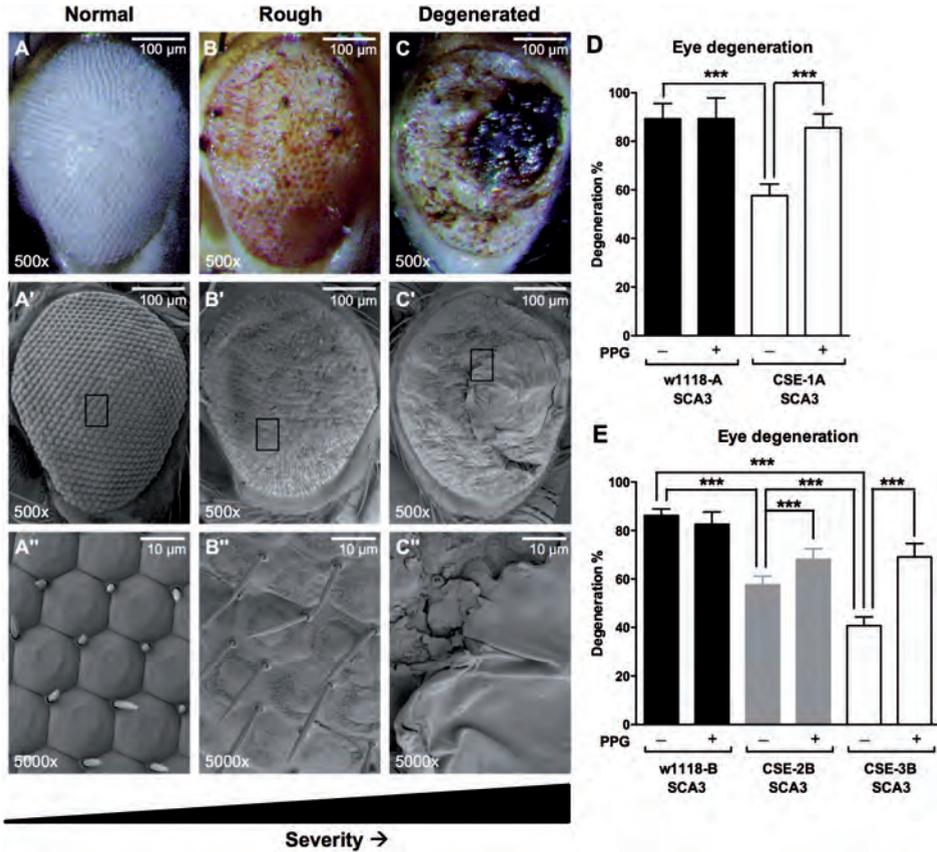
Figure 4 – Overexpression of CSE partly rescues the phenotype of SCA3 in *Drosophila*. Representative (A, B, C) light microscopy pictures with correlative (A', A'', B', B'', C', C'') scanning electron microscopy pictures of eye phenotypes. (A, A', A'') Normal wild type eye phenotype. (B, B', B'') SCA3 expressing fly with an eye phenotype classified as rough eyes. (C, C', C'') SCA3 expressing fly with an eye phenotype classified as degenerative eye. (D and E) In all three transgenic CSE overexpression lines the degree of eye degeneration was decreased compared to isogenic control lines. Inhibition of CSE by 2 mM PPG diminished this effect. For quantification, the number of rough and degenerated eyes in at least three independent experiments (n=100-300 per experiment) were counted. (E) The transgenic line with the highest degree of CSE overexpression rescued the phenotype of eye degeneration to the greatest extend. ***p<0.001, error bars indicate SEM.

be enzymatically converted to H_2S (Fig. 1).[14,31,32] STS is a stable non-toxic compound that is used in clinical practice for decades to treat caliciphylaxis, extravasations during chemotherapy or cyanide poisoning.[33] In the offspring of w1118-B flies crossed with SCA3-overexpressing flies, a dose-dependent rescue effect of the SCA3 phenotype was observed upon supplementation of STS. A concentration above 120 mM STS induced lethality (Fig. 5A). Therefore, the concentration of 80 mM was selected, and the results were further confirmed in both w1118-A and w1118-B backgrounds (Fig. 5B). These data show that the suppression of SCA3-associated degeneration is
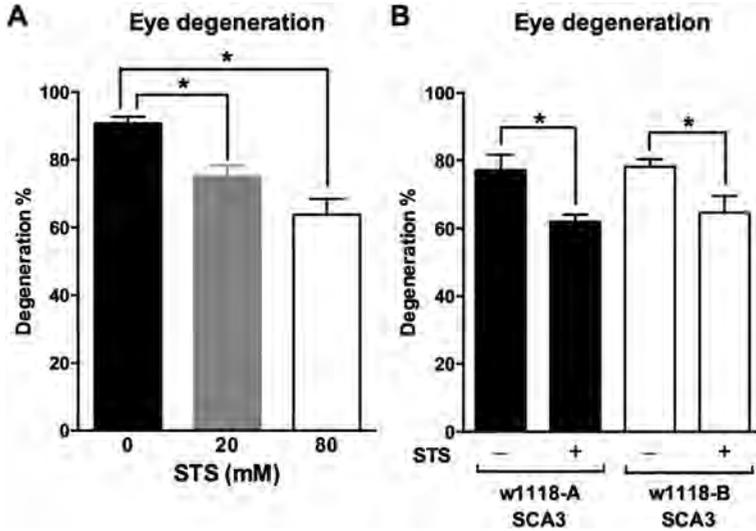
Figure 5 – **Treatment with STS suppresses SCA3-associated degeneration in** *Drosophila*. Eye degeneration was investigated with light microscopy. The number of rough and degenerated eyes was counted in three independent experiments (n=100-300 per experiment). (A) Increasing concentrations of sodium thiosulfate (STS) resulted in a reduced percentage of degenerated rough eyes in a w1118-B background. (B) Addition of 80 mM STS to the food of both different wild type lines partly rescued the phenotype of eye degeneration in the SCA3 background. *p<0.05, error bars indicate SEM.

achieved not only by activation of the transsulfuration pathway, but also by treatment with stable components of the transsulfuration pathway.

Overexpression of CSE does not induce a change in levels of insoluble proteins

Rescue of SCA3-induced tissue degeneration by CSE overexpression can be mediated by improved solubility of the aggregation-prone truncated human ATXN3 protein or by decreasing toxic effects downstream of the insoluble protein aggregates. In order to discriminate between these two possibilities, we determined ratios of insoluble versus soluble fractions of ATXN3 proteins in SCA3 flies in the absence and presence of CSE overexpression as described previously.[34] An increased insoluble/soluble ratio indicates an increase in protein aggregation. Overexpression of CSE did not significantly alter the insoluble/soluble ratio (Fig. 6, Supplementary Fig. 3), suggesting that protective effects of CSE overexpression are downstream of the formation of toxic protein aggregates.

Overexpression of CSE prevents SCA3-promoted immune induction

Several findings suggest that inflammation contributes to the multifaceted pathogenesis of SCA3 disease.[35] In *Drosophila*, Dif-Dorsal and Relish pathways are equivalent to the nuclear factor κB (NFκB) signaling in mammals, and are key factors in the induction
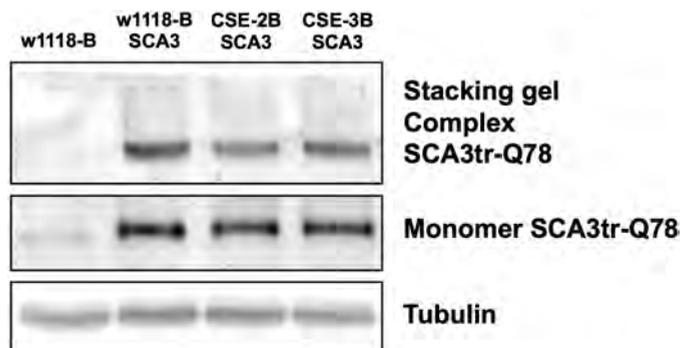
Figure 6 – Overexpression of CSE is not associated with the changes in production or aggregation of SCA3tr-Q78 protein. Heads of SCA3-overexpressing flies were analyzed for the amount of SCA3tr-78 protein and the level of its aggregation using anti-HA antibody. Tubulin was used as a loading control. In the SCA3 flies, both soluble and aggregated protein fractions could be detected. Additional CSE overexpression did not significantly alter the solubility of the SCA3 protein.

of the innate immune response.[36] To investigate whether CSE mediated protection against SCA3 is associated with reduced inflammation, we performed a qPCR analysis for antimicrobial peptides that can be activated by both immune pathways. In the Dif-Dorsal pathway immune-induced molecule 1 (IM 1), immune-induced molecule 2 (IM 2) and Drosomycin were analyzed;[37,38] in the Relish pathway, Attacin, Cecropin and Diptericin were analyzed.[36] Overexpression of the human SCA3 gene increased the immune response in both pathways. Additional CSE overexpression significantly influenced all investigated players of the Dif-Dorsal pathway (Fig. 7). A comparable effect was seen for only a subset of investigated players in the Relish pathway (Supplementary Fig. 4). As for the Dif-Dorsal pathway, the initial raise in IM 1, IM 2 and Drosomycin mRNA levels caused by SCA3 was reversed in all CSE-overexpression lines (Fig. 7). The decrease in IM 1 and IM 2 expression was associated with the degree of CSE overexpression (Fig. 7). This shows that the suppressive effect of CSE overexpression on eye degeneration in SCA3 flies is associated with a dampening of the Dif-Dorsal innate immune response pathway.

Overexpression of CSE reduces levels of oxidative damage of proteins in SCA3 flies

Oxidative stress is associated with the pathogenesis of SCA3 disease.[39,40] Previously, we have shown that CSE deficiency is linked to increased levels of oxidative stress.[41] Here, we investigated whether CSE overexpression was able to reduce increased levels of oxidative stress in a SCA3 background by using Oxyblot assays (Fig. 8). SCA3 flies showed increased levels of oxidized proteins compared with their isogenic non-SCA3 control lines. The level of oxidized proteins was strongly reduced in all three CSE overexpression lines in the SCA3 background as compared to the isogenic controls in the SCA3 background.
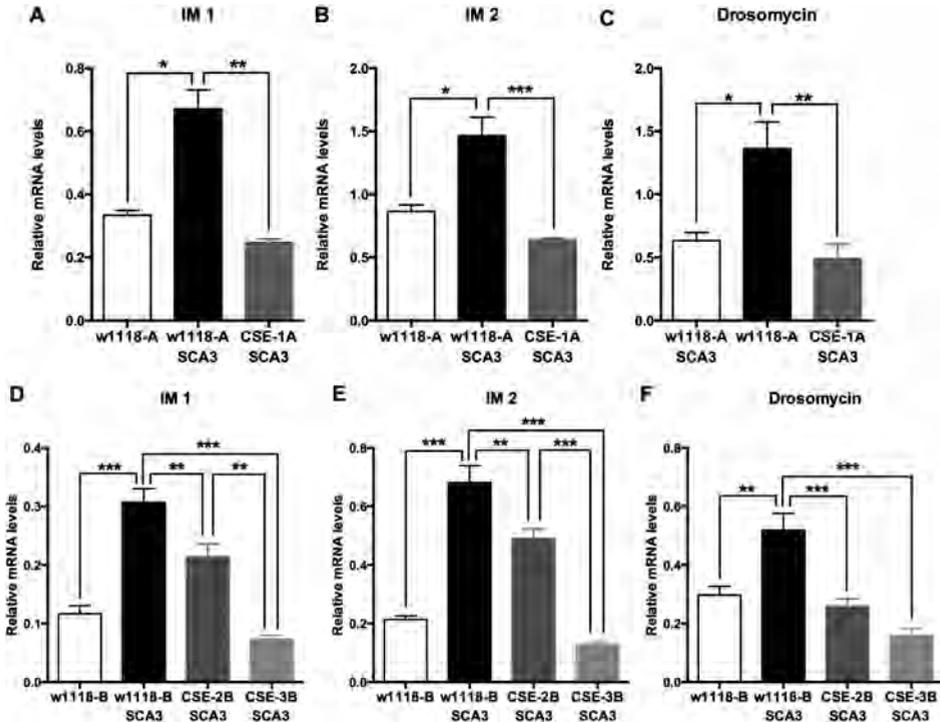
Figure 7 – **Overexpression of CSE prevents SCA3-related immune induction.** mRNA levels were determined by qPCR and in SCA3 expressing flies immune induction was observed. In CSE-1A flies, no induction of (A) IM 1, (B) IM 2 and (C) Drosomycin was observed compared to isogenic wild type flies in the SCA3 background. Similar results were observed in the other genetic backgrounds. In both the CSE-2B and CSE-3B line, there was no upregulation of (D) IM I, (E) IM 2 and (F) Drosomycin. In the CSE-3B line the mRNA levels of IM 1 and IM 2 were lower compared to the CSE-2B line. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, error bars indicate SEM.

DISCUSSION

The current study revealed that in control human brains, CSE is present in vascular endothelium, neurons and astrocytes in pontine tissue and in SCA3 patients' pontine tissue CSE localization is unaltered but the levels are reduced. Overexpression of CSE and treatment with the H_2S donor STS is protective against SCA3-related tissue degeneration in *Drosophila*. CSE overexpression in *Drosophila* is associated with reduced expression of players of the innate immune Dif-dorsal pathway and decreased levels of oxidative stress. The protective effects of CSE do not affect the formation of insoluble protein aggregates and therefore CSE-induced protection acts most likely downstream of these toxic entities.

Our data show decreased levels of CSE in pontine tissue of SCA3 patients (Fig. 2I and J). Recently, decreased levels of CSE were also demonstrated in striatal brain samples from patients with Huntington's disease.[22] It was also demonstrated that CSE-/- mice show impaired locomotor functions, therefore it is possible that low levels
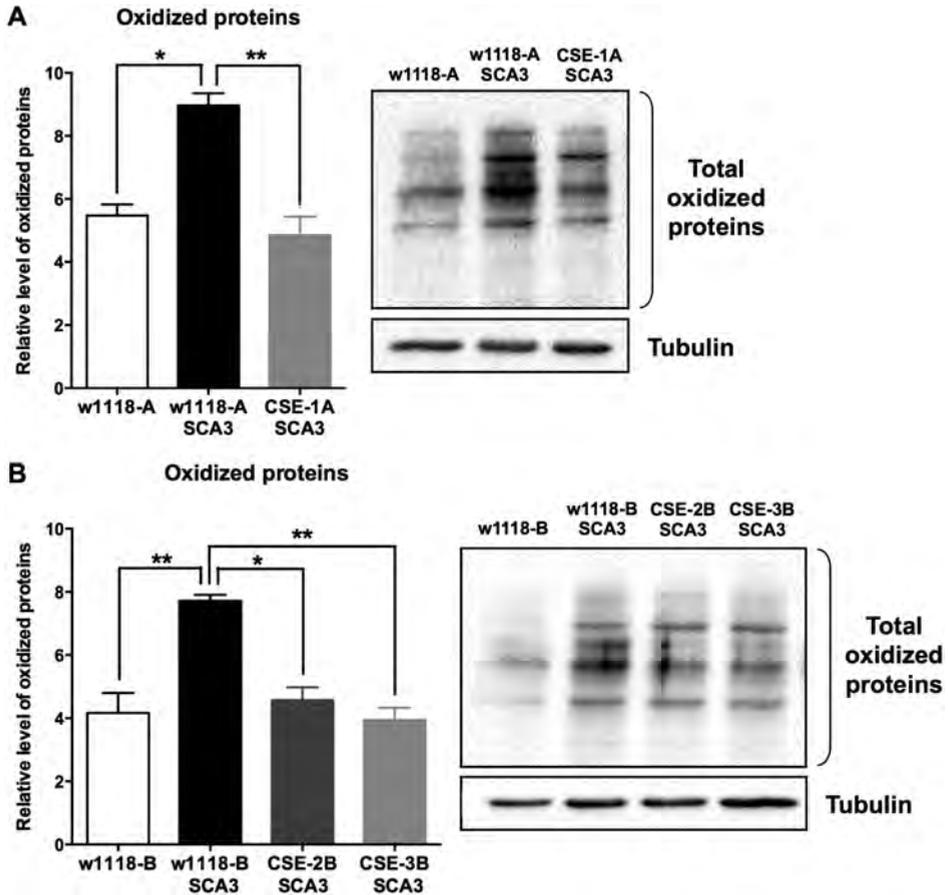
Figure 8 – Increased levels of oxidized proteins in SCA3 flies are rescued by CSE overexpression. (A and B) Oxyblot analysis revealed that SCA3 flies have higher total levels of oxidized proteins compared to wild type flies. CSE overexpression in the SCA3 background results in reduction of oxidative damage to the proteins. Total levels of oxidized proteins were normalized to tubulin. *p<0.05, **p<0.01, error bars indicate SEM.

of CSE negatively influence the development of neurodegenerative phenotypes in Huntington's disease and in SCA3. This is consistent with our findings that CSE overexpression is beneficial in the *Drosophila* SCA3 model.

CSE is an essential enzyme in the transsulfuration pathway and contributes to the endogenous production of H_2S.[11,12] Therefore, overexpression of CSE in our experiments may result in increased H_2S levels, and this may act protective against degeneration associated with protein aggregation induced by SCA3. TS, an intermediate in the transsulfuration pathway, can dynamically converted to H_2S.[14,31,32] Treatment of SCA3 flies with STS resulted in similar protective effects as CSE overexpression. This points to the possibility that the beneficial effects of CSE as demonstrated in the SCA3 flies are related to increased levels of H_2S.

To our knowledge, protective effects by CSE overexpression in neurodegenerative animal models have not been described before. However, neuroprotective effects of the gasotransmitter H_2S have been reported previously in experimental models for Parkinson's disease,[21] vascular dementia[42] and homocysteine induced neurotoxicity,[43] but also in *in vitro* models for oxidative stress in neurons[44] and Alzheimer's disease.[45] In an experimental model for Parkinson's disease, inhalation of H_2S prevents the development of neurodegeneration and movements disorders.[20] Although the exact neuroprotective action of increased levels of CSE and H_2S can not be explained yet in these models and in ours, several candidate mechanisms may contribute.

We show evidence that overexpression of CSE in flies does not suppress the formation of insoluble protein aggregates but does dampen the immune response and this in turn is protective against increased tissue loss in SCA3 expressing eyes. Firstly, inflammation has been implicated as a critical mechanism responsible for the progressive nature of neurodegeneration[35,46] and there is an inverse link between an activated transsulfuration pathway and the immune response. In experimental models, H_2S exerts anti-neuroinflammatory effects via inhibition of p38/Jun nuclear kinase and NFκB signaling pathways.[47] The inhibition of CSE by PPG leads to increased inflammation.[48] Further investigations are required to confirm whether this order of events explains the observed association between increased expression of CSE, a decreased immune activation and tissue loss protection in the presented SCA3 model.

Besides a modulating effect on the immune response, H_2S is able to facilitate the production of the chief regulator of cellular redox homeostasis GSH and scavenges ROS in mitochondria.[15,16] Furthermore, CSE has been shown to be a modulator of oxidative stress in mice.[41] SCA3 is associated with oxidative stress because mutant ATXN3 is associated with a significantly reduced capability to counteract oxidative stress that contributes to neuronal cell death in SCA3.[49] Based on this and on our observations, it is possible that overexpression of CSE results in neutralization of ROS, thereby preventing further tissue loss induced by SCA3. Protective interventions by overexpressing the molecular chaperone HSP70 also resulted in suppression of neurodegeneration in *Drosophila* overexpressing poly Q expansions without modifying protein aggregation.[24] These data and ours indicate that the modulation of effects downstream of protein aggregate formation is a possible therapeutic target. Protein S-sulfhydration is a novel posttranslational mechanism that is regulated by CSE and is involved in the modulation of protein activity.[50,51] For example, the activity of neuroprotective ubiquitin ligase parkin is regulated by sulfhydration. Parkin sulfhydration is markedly depleted in the brains of patients with Parkinson's disease.[52] This suggests that boosting of the transsulfuration pathway may contribute to neuroprotection via increased sulfhydration of specific proteins.

Our data show that CSE is endogenously expressed in SCA3 affected brain regions, therefore it may be possible to increase CSE expression by pharmacological inventions to protect against tissue degeneration in SCA3. Little is known about the regulation of CSE, but there are some substances that are able to influence CSE activity or transcription.

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There is evidence that myeloid zinc finger 1 and specificity protein 1 transcription factor affect the transcription of CSE.[53] Furthermore, studies suggest that CSE can be upregulated by bacterial endotoxin[53,54] and by nitric oxide.[55] S-adenosylmethionine and pyridoxal-5'-phosphate stimulate CSE activity to increase H_2S production.[56,57]

Our data indicate a modifying role of the transsulfuration pathway in SCA3. The presence of CSE in SCA3-relevant brain regions in humans, together with the protective effects of CSE overexpression in *Drosophila*, indicates the relevance for future research on developing clinically applicable activators of CSE or the transsulfuration pathway.

MATERIALS AND METHODS

Below we provide a brief overview of the methods used for experiments presented in this article. For further details, please see *Supplementary Materials and Methods*.

Drosophila stocks

As wild-type control the y1w1118 *Drosophila* line was used. Eip55E (*Drosophila* CSE)-overexpressing lines were generated in the laboratory. The GMR-GAL4 SCA3trQ78 fly stock was a kind gift from Prof. Bonini.[24,29] The detailed description of the *Drosophila* lines and flies food, backcrossing and supplementation of chemical compounds information can be found in *Supplementary Materials and Methods.*

Eye degeneration assay

To evaluate relative degeneration, we used an eye scoring method that was previously described.[58,59] Irregularly-structured depigmented eyes without dark patches were defined as rough. The presence of one or more black patches along with the irregular structure and depigmentation was considered a degenerated rough eye. Each eye of one-day-old flies was scored as a singular entity. We scored the total amount of degenerated eyes as opposed to the total amount of eyes (rough + degenerated). Total count of eyes scored per condition was between 100 and 1000 depending on the number of progeny of a particular phenotype.
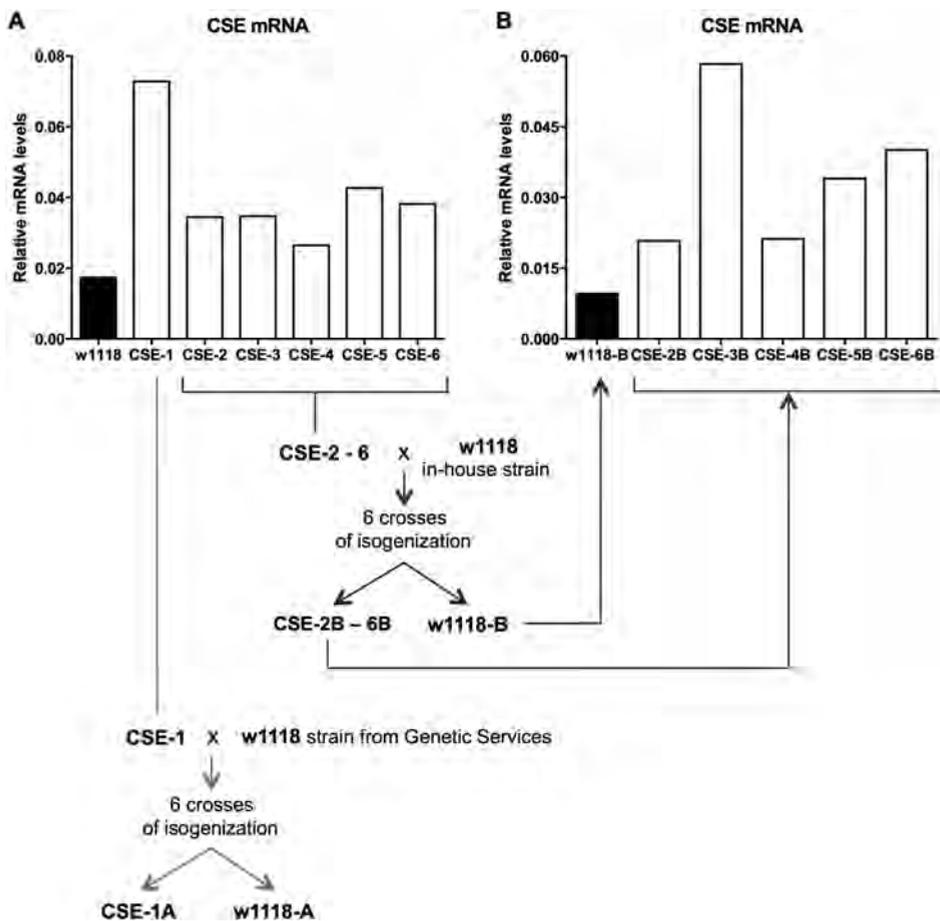
Molecular biology techniques

For the detailed description of quantitative RT-PCR, Western blot and Protein oxidation analyses, Immunohistochemistry that are used in the current study, please see *Supplementary Materials and Methods.*

ACKNOWLEDGEMENTS

SUPPLEMENTARY DATA

Supplementary Figure 1 - CSE mRNA levels of different CSE overexpression lines and schematic representation of backcrossing of relevant CSE lines. (A) Relative CSE mRNA levels in fly lines received from Genetic Services and (B) in lines after backcrossing for 6 generations into the in-house w1118 strain expressed under control of the daughterless driver. Below: scheme of crosses to isogenize fly lines with 2 different w1118 control strains.
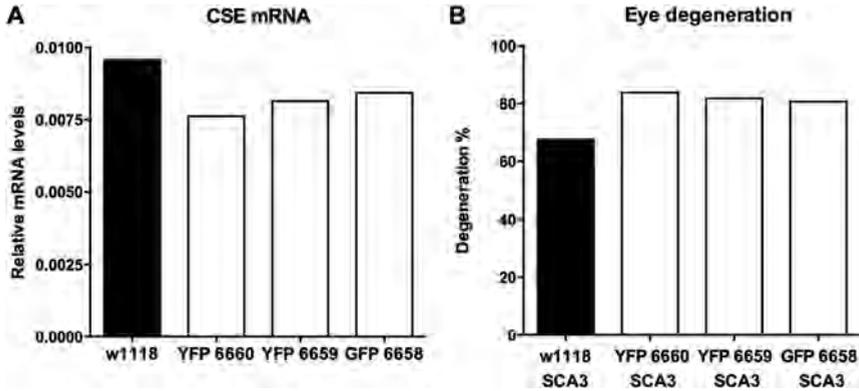
Supplementary Figure 2 – Suppression of SCA3 by CSE overexpression is not caused by a titration of GAL4. (A) The GFP line and both YFP lines have comparable and, if not, lower levels of CSE mRNA compared to their isogenic wild type control. (B) In a SCA3 background, the level of eye degeneration in the GFP line and both YFP lines is similar or higher compared to their isogenic wild type control line.

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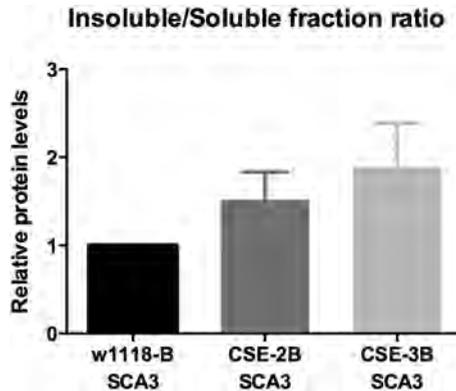
Supplementary Figure 3 – Overexpression of CSE in a SCA3 background is not associated with a decrease in insoluble/soluble fraction ratio of SCA3tr-78 protein. Quantification of ratio between the relative intensity of the protein in the stacking gel and SCA3tr-78 monomer band. There is no significant change in the protein solubility upon the overexpression of CSE in a SCA3 background.
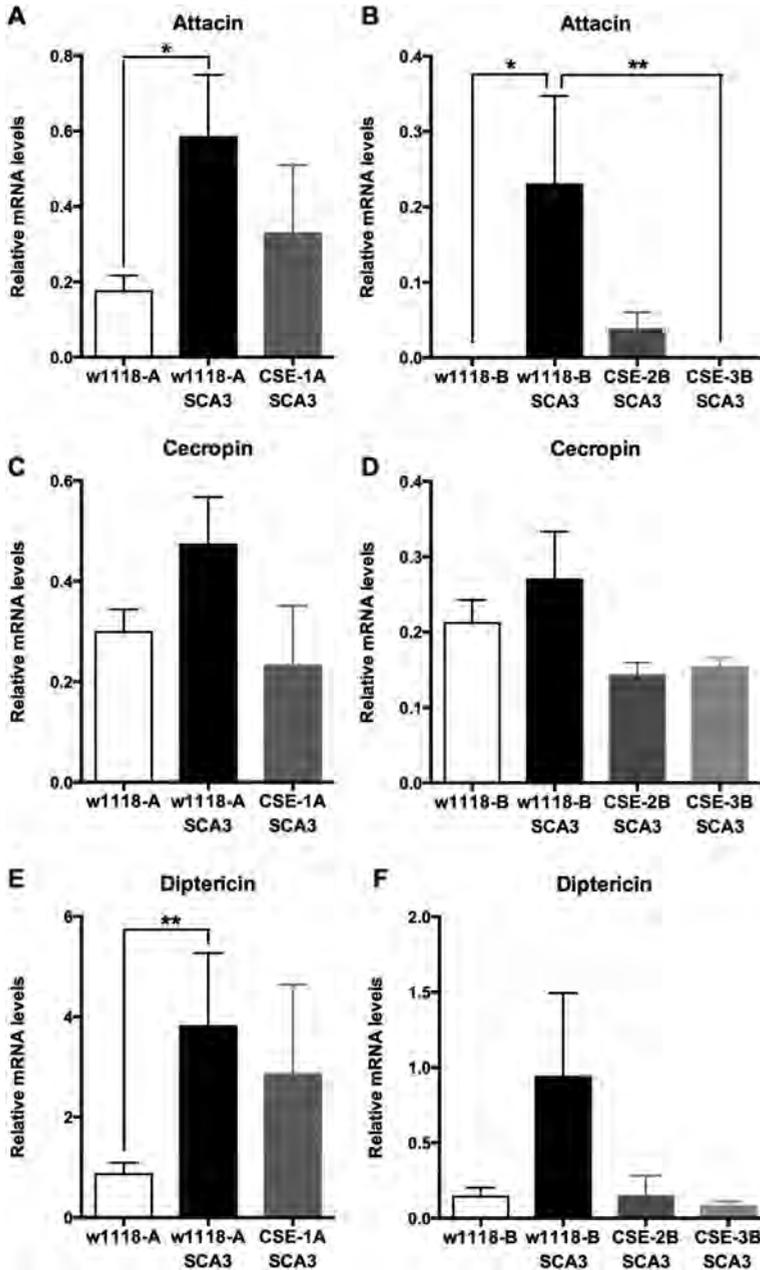
Supplementary Figure 4 – Inflammation genes that are variously affected by CSE overexpression in a SCA3 background. Expression of inflammation genes from the Relish pathways upon the overexpression of SCA3 alone or in combination with CSE. (A and B) Attacin is induced by SCA3 but not differently expressed in the CSE-1A line. (B) In the CSE-3B line Attacin mRNA levels are reduced compared to w1118-B SCA3. (C and D) Cecropin is not significantly influenced by CSE overexpression. (E) Diptericin is significantly induced in the w1118-A background but not in the (F) w1118-B background. (E and F) In all CSE overexpression lines mRNA levels of Diptericin are not different from the isogenic control lines.

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SUPPLEMENTARY MATERIALS AND METHODS

Collection of human pontine tissue

Brains from 7 genetically confirmed SCA3 patients and 7 controls without medical histories of neuropsychiatric diseases were analyzed (table S1). Immediately after brain autopsy samples (15x15x5mm) of the base of the pons were snap frozen. Informed consent was obtained from all SCA3 patients. Control cases were anonymized and coded according to the National Code for Good Use of Patient Material. All procedures were approved of and in accordance with the Medical Ethical Committee of the University Medical Center Groningen.

Immunohistochemistry for CSE

For immunostaining, frozen human pontine sections were dried and fixed in acetone. Subsequently, sections were incubated at room temperature with the primary antibody (Proteintech rabbit polyclonal CSE antibody 11217-1-AP (1:500)) for 60 minutes. Endogenous peroxidase was blocked with H_2O_2 in phosphate buffered saline (PBS, pH 7.4) for 30 minutes. Binding was detected using sequential incubation with a peroxidase-labeled secondary antibody (Dakopatts, Glostrup, Denmark) for 30 minutes. All antibodies were diluted with PBS supplemented with 1% BSA. At the secondary antibody dilution, 1% human AB serum was added. Peroxidase activity was developed using filtered 3-amino-9-ethylcarbazole for 15 minutes containing H_2O_2. Counterstaining was performed using Mayer's hematoxylin. Appropriate isotype and PBS controls were consistently negative.

Drosophila stocks

For overexpression of the genes, the UAS-GAL4 binary system for targeted gene expression was used (1). Eip55E (*Drosophila* CSE) cDNA was cloned into the pUAST vector and verified by sequencing. Transgenic CSE fly lines were generated by Genetic Services Inc. (Sudbury, USA) in a w1118 (y1w1118) background by random insertion into the genome. Fly stocks bearing the CSE transgene were backcrossed into control lines for six generations. CSE-1A was isogenized with the w1118-A line; and CSE-2B and CSE-3B were isogenized with the w1118-B line. Transgenes were selectively overexpressed in the eyes by using GMR-GAL4 driver flies (stock #1104) and ubiquitously using the daughterless driver flies (stock #8641). To test whether a titration effect existed, a UAS-GFP (stock #6658) and two UAS-YFP (stock #6659; #6660) lines were used. The driver stocks and stocks bearing fluorescent proteins under the control of UAS were ordered from Bloomington Drosophila stock center (Indiana University, USA). The GMR-GAL4 SCA3trQ78 fly stock was a kind gift from Prof. Bonini. All crosses were performed at 25°C according to standard protocols. For all the experiments only male flies were used.

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Fly food and supplementation of PPG and STS

Fly strains were raised and crossed on Nutri-Fly Bloomington food (Brewer's Yeast, Sucrose, Agar Type II, Glucose, Yeast Extract, $MgSO_4$ x $6H_2O$, Peptone, $CaCl_2$ x $2H_2O$; Genesee Scientific). For inhibition of CSE, fly crosses were set up on propargylglycine (PPG)- supplemented food. PPG (Sigma, Zwijndrecht, the Netherlands) was added to freshly prepared fly food to a final concentration of 2 mM as previously described (2). In the same manner, STS (Sigma, Zwijndrecht, the Netherlands) was administered to the fly food reaching final concentrations of 20 mM, 80 mM and 120 mM. Every two days, the relevant concentration of STS dissolved in distilled water was added to the vials during development of the flies.

Light and electron microscopy

To enable correlative analysis, the same fly for each condition was used in both light microscopy (LM) and scanning electron microscopy (SEM). One-day-old flies were decapitated, heads were dehydrated through ethanol series, and after acetone as an intermediate step, air dried from tetramethylsilane (Sigma-Aldrich). Light microscopy images of fly eyes were taken with a Leica M165 FC stereo microscope followed by focus stacking using Adobe Photoshop. For the scanning electron microscopy, the same eyes were gold/paladium-coated (3 nm) and analyzed with a Zeiss Supra 55 SEM at 2KV using the SE2 detector.

Quantitative RT-PCR

Human pontine tissue was homogenized in lysis buffer and total RNA was extracted using the RNeasy Mini Kit (Qiagen). To verify CSE overexpression in transgenic *Drosophila* lines, total RNA was isolated from 10-15 one-day old flies using RNeasy Mini Kit (Qiagen). For each genotype/treatment, at least three independent extractions were prepared. cDNA was synthesized using Superscript II with random hexamer primers (Invitrogen, Carlsbad, USA). Gene expression was determined by quantitative real time-PCR (qPCR) using a SYBR green mastermix (iQ SYBR GREEN Supermix; Bio-Rad). Rp49 and TBP were used as normalization reference house-keeping genes for *Drosophila* and human pontine tissue, respectively. The PCR profile consisted of 15 min at 95°C, followed by 40 cycles with heating of 95°C for 15 s and cooling to 60°C for 1 min. To detect mRNA levels of immune response genes, the same procedure was followed with the exception that ten-day-old flies were used. All samples were normalized to their house-keeping gene, the average Ct values for target genes were subtracted from the average housekeeping gene Ct values to yield the delta Ct. Results were expressed as $2^{-\Delta Ct.}$ The following primers were used:

Western blot analysis

Human pontine tissue was homogenized in RIPA buffer (Sigma, Zwijndrecht, the Netherlands) supplemented with protease inhibitor cocktail (Sigma, Zwijndrecht, the

Supplementary table 1 - Primers used in the experiments

Gene	Primer
Drosophila CSE (Eip55E)	For - TGAACGGTCATACGGATGTG
	Rev - ACCTGATAGCAGTCGAATGG
Drosophila rp49	For - GCACCAAGCACTTCATCC
	Rev - CGATCTCGCCGCAGTAAA
Drosophila IM1	For - TGCCCAGTGCACTCAGTATC
	Rev - GATCACATTTCCTGGATCGG
Drosophila IM2	For - AAATACTGCAATGTGCACGG
	Rev - ATGGTGCTTTGGATTTGAGG
Drosophila Drosomycin	For - GTACTTGTTCGCCCTCTTCG
	Rev - GATTTAGCATCCTTCGCACC
Drosophila Diptericin	For - ACCGCAGTACCCACTCAATC
	Rev - ACTTTCCAGCTCGGTTCTGA
Drosophila Attacin	For - GCTTCGCAAAATAAACTGG
	Rev - TCCCGTGAGATCCAAGGTAG
Drosophila Cecropin	For - GAACTTCTACAACATCTTCGT
	Rev - TCCCAGTCCCTGGATTGT
Human CSE	Assay-on-Demand Applied Biosystems ID: Hs00542284_m1
Human TBP	For - GCCCGAAACGCCGAATAT
	Rev - CCGTGGTTCGTGGCTCTCT

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Netherlands). Protein concentrations were determined using the pyrogallol red molybdate method (3). Equal amounts of protein were loaded onto 12.5% SDS-polyacrylamide gels. Proteins were transferred from the gels onto nitrocellulose membranes and immunostained. Aspecific binding of the antibodies was prevented using 5% milk in Tris-buffered saline-0.1% Tween-20 (TBST). Membranes were incubated with the primary antibody solution in 5% milk in TBST overnight at 4 °C (for CSE: Proteintech rabbit polyclonal CSE antibody 11217-1-AP (1:1000), for GAPDH: mouse monoclonal GAPDH antibody, Fitzgerald Industries (1:50 000)). Afterwards, membranes were incubated with a secondary antibody (for CSE: goat anti-rabbit IgG horseradish peroxidase (1:500) (Dakopatts, Glostrup, Denmark), for GAPDH: anti-mouse IgG horseradish peroxidase (1:5000), (GE Healthcare UK Limited)) in 5% milk in TBST. All antibody incubations were followed by washing with TBST. The membranes were incubated for 4 minutes with ECL western blot substrate (Pierce, Rockford, USA) and placed in an autoradiography cassette and developed in a dark room. The films were placed in the developer (Sigma, Zwijndrecht, the Netherlands) for 2 minutes and subsequently washed in water and fixed in the fixer (Sigma, Zwijndrecht, the Netherlands). Intensity of CSE bands was measured using ImageJ and normalized to the intensity of GAPDH bands.

For the SCA3tr-78 insoluble/soluble fraction analysis, one-day-old flies were directly frozen in liquid nitrogen and decapitated. Twenty-five heads per condition were homogenized in Laemmli Sample Buffer (62,5 mM Tris/HCL pH 6,8; 2% SDS; 10% glycerol; bromophenol blue) containing 2% beta-mercaptoethanol. Ten ul of each sample was loaded onto 12.5% SDS-polyacrylamide gels. Further procedures were similar to those for human CSE protein detection. As a primary antibody to detect SCA3tr-78 rat monoclonal high affinity anti-HA-peroxidase (1:500) (clone 3F10; Roche, Indianapolis, USA) was used. For tubulin detection mouse anti-tubulin (Sigma, Zwijndrecht, the Netherlands) was used. Goat anti-rat IgG horseradish peroxidase (1:5000) (GE Healthcare UK Limited) was used as a secondary antibody for SCA3tr-78 detection. As a secondary antibody for tubulin detection, sheep anti-mouse IgG horseradish peroxidase (1:4000) (GE Healthcare UK Limited) was used. To calculate the ratio between the SCA3tr-78 insoluble and soluble fraction, the total intensity of the signal in the stacking gel normalized to tubulin was divided by the intensity of SCA3tr-78 monomer band normalized to tubulin. Western Blots from three independent experiments with unique sets of samples were used for quantification using ImageJ.

Protein oxidation detection

Ten-days-old flies were directly frozen in liquid nitrogen and decapitated. Per condition, 15 heads were homogenized in RIPA buffer containing 2% beta-mercaptoethanol. Protein oxidation was assessed with OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, USA) according to manufacturer's instructions. As a loading control, blots were immunostained with a rabbit alpha-tubulin (Sigma, Zwijndrecht, the Netherlands) antibody. The amount of oxidized proteins was measured by comparing chemiluminescence of the samples normalized to alpha-tubulin levels.

Statistical analysis

Data were analyzed using GraphPad Prism 5.0 and IBM SPSS 20.0 software. Normality was tested using the Kolmogorov-Smirnov test. The unpaired Student's t-test was used for comparisons between 2 groups with normal distribution. Non-parametric data were compared using the Mann-Whitney U-test. For comparisons between three groups, an ANOVA with Bonferroni post-test was used for parametric data and a Kruskal Wallis with Dunnett's post-test was used for non-parametric data. For the analysis of the number of degenerated eyes, a logistic regression was used. The number of replications of each experiment was at least three. Statistical significance was accepted at $p < 0.05$. All data are expressed as the mean ± standard error of the mean (SEM) unless indicated otherwise.

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